

# STUDIES IN THE BIOLOGY OF THE LEECH<sup>1</sup>

## VII. A NEW METHOD OF STAINING NERVOUS TISSUE

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The technique presented in this paper employs certain elements of the original Golgi method, combined with the working principle of the reduced silver method of Cajal. The accompanying illustrations demonstrate how effectively this technique differentiates the histological as well as the cytological details of the nervous system of the leech.<sup>1</sup> This method has in common with that of Golgi's the useful character of staining only some of the neurons. The stained portions are impregnated with the reduced silver compound showing clearly their structure and pattern. The nerve cells and their processes are black against a light blue background. Muscle or connective tissue cells, where appearing in the same field, are tinted red. If a counter stain is used color variations will be noted.

### MILLER'S METHOD, VARIANTS A AND B

#### DETAILS OF PROCEDURE

##### 1. *Relaxing.*

The leech was placed in a shallow dish containing a 0.1 per cent chloretone solution to which had been added a pinch of protoleptic enzyme. Caroid was found to be satisfactory. This latter, while not absolutely necessary, materially aided in disposing of the copious mucous secreted by the organism. After five minutes in the chloretone solution, there was added drop by drop, a saturated solution of the same until the leech was completely relaxed. It was then placed in a wax bottom dissecting pan. Here the leech was pinned to normal size and received the first application of the killing and fixing agent.

##### 2. *Killing and Fixing.*

A solution of 10 per cent formalin was used as a killing and fixing medium. The usual time of fixation was twenty-four hours. The animal was then washed thoroughly in tap water and in several changes of distilled water.

##### 3. The leech was dissected at this point. A ventral strip of body wall was frequently left attached for orientation. A completely dissected nerve trunk was pinned on a strip of heavy paper bent to form a bow.

##### 4. The tissue was then immersed in 3 per cent potassium dichromate for two weeks, then

##### 5. Rinsed in several changes of distilled water.

##### 6. The tissue was then placed in 1 per cent solution of silver nitrate; after a precipitate formed the tissue was transferred to a fresh solution of 1 per cent silver nitrate for 1 week.

##### 7. Rinsed in several changes of distilled water.

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<sup>1</sup>*Haemopsis marmoratis* (Say).

8. *Dehydration.*

In the dehydration of material for celloidin imbedding it was necessary to insure absolute removal of all water. The following procedure proved to be adequate for parts of the nervous system of small sections of the animal. Beginning with 30 per cent alcohol the material was carried through 50-70-80-95-100 per cent alcohol for a minimum of two hours each. Two changes of two hours each in absolute alcohol-ether completed the process.

9. *Infiltration and Embedding.*

Following dehydration the tissue was placed in 10 per cent celloidin for eight hours and left overnight in 15 per cent celloidin. The material to be mounted was supported by a block of hardened celloidin and oriented within a mould formed by surrounding the fiber cutting block with gum paper. The cutting block mould was then filled with 15 per cent celloidin; the tissue oriented and hardened under a bell jar. Chloroform was used to complete the gardening process. When completely hardened the block was placed in thin cedar wood oil where it remained until sectioned. (In the procedure outlined above the block of tissue did not exceed one centimeter in length.)

10. Sections were cut to desired thickness. (Cedar wood oil was used in preference to alcohol during the cutting process.)
11. Sections were placed in thin cedar wood oil, where they were kept until ready to proceed with the next step. Sections were kept serially by separates. At this point two alternative procedures were followed.

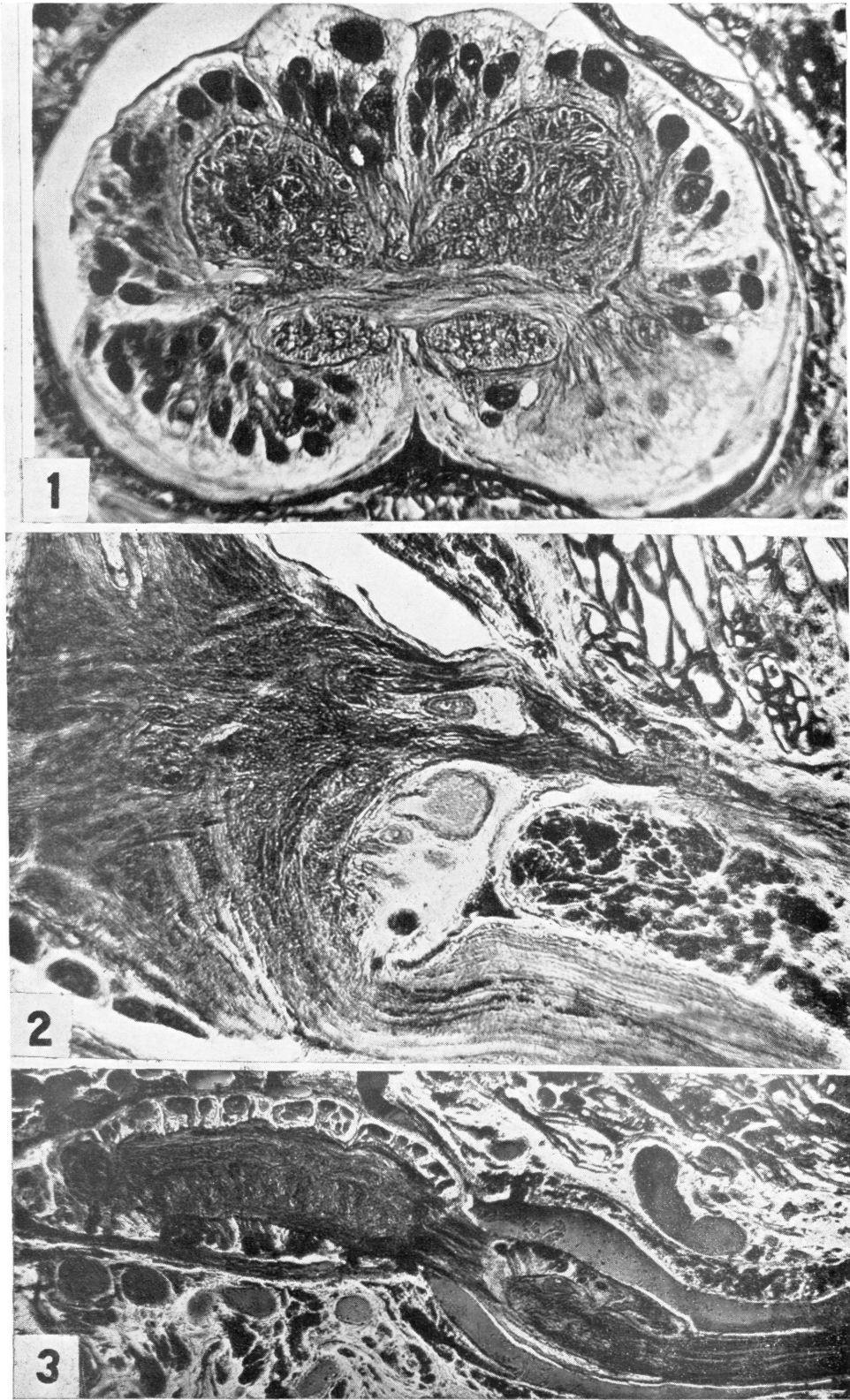
## VARIANT A

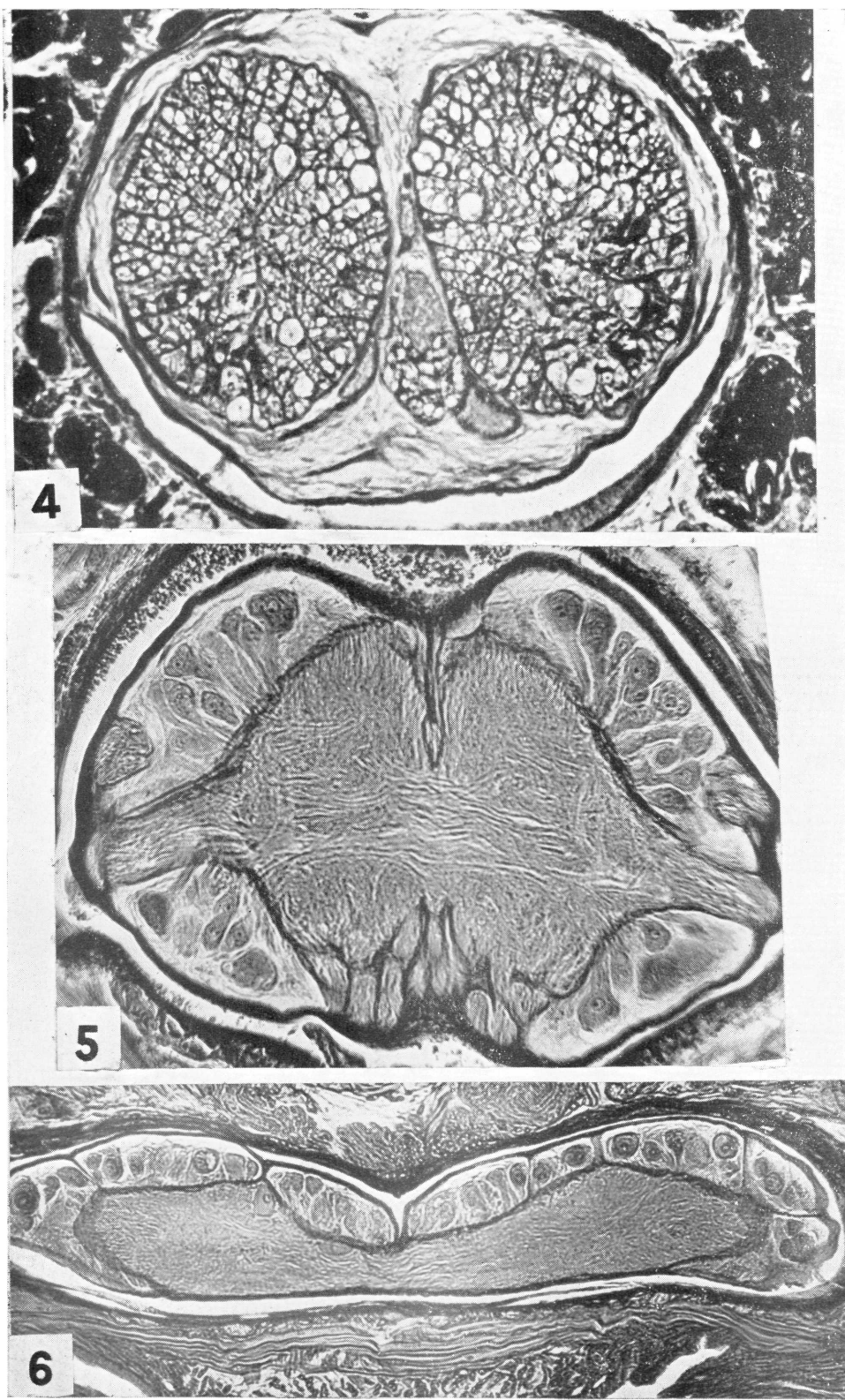
- 12A. Sections gradually hydrated and placed in
- 13A. Six per cent silver nitrate for four days (in the dark).
- 14A. Washed in several changes of distilled water.
- 15A. Reduced in the following mixture for 24 hours: (In the dark.)
 

Hydroquinone.....	2.0 g.
Neutral formalin.....	20.0 cc.
Water (distilled).....	200.0 cc.
- 16A. Washed in several changes of distilled water.
- 17A. (Optional). Tone in 0.2 per cent gold chloride, 30 minutes.
- 18A. Washed in distilled water.
- 19A. Fixed in 5 per cent hypo-sulphite of soda, 30 seconds.
- 20A. Washed in distilled water.
- 21A. (Optional). Counter stain at this point if desired.
- 22A. Dehydrate, clear, mount in clarite. (Follow standard procedures which will vary according to type of counter stain used.)

## EXPLANATION OF PLATE I

- FIG. 1. Photomicrograph—Method A. Cross-section through a ganglion.  
 FIG. 2. Photomicrograph—Method A. Frontal section through a ganglion.  
 FIG. 3. Photomicrograph—Method A. Longitudinal section through the caudal ganglion.





## VARIANT B

- 12B. Sections from cedar wood oil were gradually hydrated and placed in
- 13B. 20 per cent silver nitrate solution 60-90 minutes.
- 14B. Washed quickly in distilled water and placed in
- 15B. Ammoniated silver nitrate, 3 minutes.

The above solution was prepared by adding concentrated aqua ammonia, drop by drop, to 20 per cent silver nitrate solution until the precipitate is almost dissolved. The mixture was then filtered. (The ammoniated silver nitrate must be freshly prepared and can be used only once.)

- 16B. Washed quickly in distilled water.
- 17B. Sections were placed in 10 per cent formalin one minute.
- 18B. Washed in several changes of distilled water.
- 19B. Toned in 2.0 per cent gold chloride 10 minutes.
- 20B. Washed in distilled water.
- 21B. (Optional procedure.) Reduce in 2.0 per cent oxalic acid + 1 cc. of formalin per 100 cc. of solution, 20 seconds.
- 22B. Washed in distilled water.
- 23B. Fixed in 5.0 per cent of hypo-sulphite of soda, 2 minutes.
- 24B. Washed in distilled water.
- 25B. (Optional.) Counter stain at this point if desired.
- 26B. Dehydrate, clear, mount in clarite. (Follow standard procedures which will vary according to type of counter stain used.)

Counter staining was successfully employed following either of the above procedures. Mayer's acid hematoxylin and eosin or haemalum gave good cellular differentiation. Mallory's phosphotungstic acid hematoxylin was very effective following either of the above variations. Follow standard procedures for counter staining and subsequent treatment.

Method A produced the best material for histological study, particularly noted in the longitudinal and frontal sections.

Method B was especially useful in cytological study, being particularly valuable in the study of ganglion cells.

The writer is preparing a manuscript of "Modifications in Neurological Micro-technique," as applied to leech material. This paper will appear in an early issue of this Journal.

## REFERENCES

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- Lee, Bolles.** 1937. The Microtometist's Vade-Mecum Edit. Gatenby and Painter, 10th Edition, Churchill, London.
- McClung, C. E. and others.** 1937. Handbook of Microscopical Technique, 2nd Revised Edition. Hoeber, New York.

## EXPLANATION OF PLATE II

- FIG. 4. Photomicrograph—Method B. Cross-section through a nerve cord.
- FIG. 5. Photomicrograph—Method B. Cross-section through a ganglion.
- FIG. 6. Photomicrograph—Method B. Cross-section through the dorsal cephalization.